

## The Somatostatin Analogue Octreotide Confers Sensitivity to Rapamycin Treatment on Pituitary Tumor Cells

Vesna Cerovac<sup>1</sup>, Jose Monteserin-Garcia<sup>1</sup>, Hadara Rubinfeld<sup>3</sup>, Michael Buchfelder<sup>4</sup>, Marco Losa<sup>5</sup>, Tullio Florio<sup>6</sup>, Marcelo Paez-Pereda<sup>2</sup>, Günter K. Stalla<sup>1</sup>, and Marily Theodoropoulou<sup>1</sup>

### Abstract

Rapamycin and its analogues have significant antiproliferative action against a variety of tumors. However, sensitivity to rapamycin is reduced by Akt activation that results from the ablative effects of rapamycin on a p70 S6K-induced negative feedback loop that blunts phosphoinositide 3-kinase (PI3K)-mediated support for Akt activity. Thus, sensitivity to rapamycin might be increased by imposing an upstream blockade to the PI3K/Akt pathway. Here, we investigated this model using the somatostatin analogue octreotide as a tool to decrease levels of activated Ser<sup>473</sup>-phosphorylated Akt (pAkt-Ser<sup>473</sup>) in pituitary tumor cells that express somatostatin receptors. Octreotide increased levels of phosphorylated insulin receptor substrate-1 that were suppressed by rapamycin, subsequently decreasing levels of pAkt-Ser<sup>473</sup> through effects on phosphotyrosine phosphatase SHP-1. Octreotide potentiated the antiproliferative effects of rapamycin in immortalized pituitary tumor cells or human nonfunctioning pituitary adenoma cells in primary cell culture, sensitizing tumor cells even to low rapamycin concentrations. Combined treatment of octreotide and rapamycin triggered G<sub>1</sub> cell cycle arrest, decreasing E2F transcriptional activity and cyclin E levels by increasing levels of p27/Kip1. These findings show that adjuvant treatment with a somatostatin analogue can sensitize pituitary tumor cells to the antiproliferative effects of rapamycin. *Cancer Res*; 70(2); 666–74. ©2010 AACR.

### Introduction

The phosphoinositide 3-kinase (PI3K)/Akt pathway is frequently overactivated in a variety of tumors either by mutations or by growth factors (1). It plays a crucial role in tumor formation and progression by controlling cell growth, proliferation, apoptosis, and metastasis (2, 3). Growth factor-bound tyrosine kinase receptors with the help of adaptors, such as insulin receptor substrate-1 (IRS-1), recruit the PI3K regulatory subunit p85 and activate PI3K and, subsequently, PDK1, Akt, and the mammalian target of rapamycin (mTOR).

Rapamycin is a mTOR inhibitor with potent immunosuppressant and antiproliferative properties. Rapamycin and its analogues (rapalogs) are effective against solid tumors presenting with overactivated PI3K/Akt pathway (4, 5). In

contrast, rapalogs displayed modest efficacy in clinical trials (6). Analysis of breast cancer biopsies derived from patients treated with RAD001 revealed increased Akt phosphorylation (7). The mTOR target p70 S6K phosphorylates IRS-1 at serine residues, leading to its dissociation from tyrosine kinase receptors and inhibition of the PI3K signaling (8–11). Rapamycin and its analogues by inhibiting mTOR decrease p70 S6K serine phosphorylation and abolish this inhibitory feedback loop to IRS-1, resulting in the observed increased Akt phosphorylation (7, 12, 13). Therefore, cotreatment with an agent able to decrease Akt phosphorylation could overcome rapamycin-induced Akt phosphorylation upstreams and improve the antiproliferative action of rapamycin analogue treatment.

Somatostatin receptor type 2 (Sst2) was shown to deactivate the PI3K pathway by inhibiting p85 tyrosine phosphorylation and decreasing Akt phosphorylation (14, 15). Somatostatin analogues targeting Sst2, such as octreotide and lanreotide, are frequently used for the treatment of neuroendocrine tumors (16).

In the present study, addition of the somatostatin analogue octreotide to rapamycin increased serine-phosphorylated IRS-1 levels, decreased rapamycin-induced pAkt-Ser<sup>473</sup> levels, and enhanced the antiproliferative effect of rapamycin in immortalized pituitary tumor cells and in human nonfunctioning pituitary adenomas (NFPA) in primary cell culture by upregulating p27/Kip1.

### Materials and Methods

**Reagents.** Cell culture materials were purchased from Life Technologies, Nunc, and Sigma. Octreotide was provided by

**Authors' Affiliations:** <sup>1</sup>Department of Endocrinology, Max Planck Institute of Psychiatry; <sup>2</sup>Affectis Pharmaceuticals, Munich, Germany; <sup>3</sup>Institute of Endocrinology and Felsenstein Medical Research Center, Tel-Aviv University, Tel-Aviv, Israel; <sup>4</sup>Department of Neurosurgery, University of Erlangen, Erlangen, Germany; <sup>5</sup>Neurosurgical Department, Hospital San Raffaele, Milan, Italy; and <sup>6</sup>Department of Oncology, Biology and Genetics, University of Genova, Genova, Italy

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

V. Cerovac and J. Monteserin-Garcia contributed equally to this work.

**Corresponding Author:** Marily Theodoropoulou, Department of Endocrinology, Max Planck Institute of Psychiatry, Kraepelinstrasse 10, 80804 Munich, Germany. Phone: 49-8930622292; Fax: 49-8930622605; E-mail: marily@mpipsy.kl.mpg.de.

doi: 10.1158/0008-5472.CAN-09-2951

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the American Peptide Co.; phorbol 12-myristate 13-acetate, MG132, and rapamycin were from Sigma. Lithium chloride and SB415286 were from Calbiochem. Octreotide was dissolved in 0.01 mol/L acetic acid, and rapamycin and SB415286 were dissolved in DMSO.

**Tumors.** This study was approved by the Max Planck Institute ethics committee, and informed consent was received from each patient or from their relatives. Twenty-eight NFPA's were included. The tumors were removed through transsphenoidal surgery and diagnosed by clinical, biochemical, radiological, and surgical findings.

**Cell culture.** Postsurgical specimens were washed with HDB buffer [15 mmol/L HEPES (pH 7.4), 137 mmol NaCl, 5 mmol/L KCl, 0.7 mmol/L  $\text{Na}_2\text{HPO}_4$ , 10 mmol/L glucose, 2.5 mg/mL amphotericin B,  $10^5$  units/L penicillin/streptomycin] and enzymatically dispersed as previously described (17). D-Valine was used to suppress the growth of contaminating fibroblasts (17). Cell viability over 80% was acceptable, as determined by the acridine orange and ethidium bromide staining. Cells were seeded in 48-well plates (100,000 per well) and left for 48 h.

The immortalized pituitary tumor cell lines GH3 and AtT-20 (American Type Culture Collection) were used. Cells were cultured in DMEM supplemented with 10% FCS, 2 mmol/L glutamine, 0.5 mg/L partricin, and  $10^5$  units/L penicillin-streptomycin at 37°C and 5%  $\text{CO}_2$ .

**Proliferation studies.** [ $^3\text{H}$ ]thymidine incorporation ([ $^3\text{H}$ ]TdR) was performed as previously described (18). Cells were treated with rapamycin, octreotide, and their combination at the indicated times for each experiment concentrations in the presence of [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci/mL}$ ) for 24 h. Cells treated with the carriers in which octreotide and rapamycin were dissolved were used as control. Cell proliferation was also assessed using the nonradioactive colorimetric WST-1 assay (Roche Molecular Biochemicals) as previously described (19). All treatments were carried out in quadruplicates.

**Colony formation.** Anchorage-independent colony formation was determined as previously described (19). AtT-20 cells were treated for 24 h, suspended in 0.3% melted agar, and plated on 0.5% agar. The cultures were incubated for 20 d and stained with MTT to show the presence of viable cells under all treatments at the end of the experiment.

**Flow cytometry.** Cell cycle was analyzed by flow cytometry after propidium iodide staining as previously described (20). Cells were serum deprived for 24 h and treated for 24 h with octreotide, rapamycin, and their combination. The vehicle in which the substances were dissolved was used as control. Analysis was done in FACSCalibur (Becton Dickinson) using the CellQuest and ModFit softwares.

**RNA extraction and semiquantitative reverse transcription-PCR.** RNA was extracted by the guanidinium isothiocyanate-phenol method. PCR for  $\beta$ -actin was performed on 1  $\mu\text{g}$  RNA to exclude genomic DNA contamination. RNA (1  $\mu\text{g}$ ) was reverse transcribed using random hexanucleotides and reverse transcription-PCR (RT-PCR) was performed for p27/Kip1 (sense: 5'-aggcgagatagcagtgagcag-3', antisense: 5'-ctggacactgctccgctaacc-3') and  $\beta$ -actin (internal control) under restrictive conditions as previously described (21). Each RT-PCR was performed in RNA extracted from three independent experiments.

**Western blot.** AtT-20 cells were treated with 1 nmol/L octreotide, 1 nmol/L rapamycin, and their combination for the times indicated for each experiment. DMSO was added to the control (untreated) and single octreotide treatment. Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer supplemented with protease and phosphatase inhibitor cocktail (Roche). Samples were separated by PAGE and blotted using standard procedures (22).

Primary antibodies were against cyclin D1, cyclin D3, cyclin E, cyclin-dependent kinase (CDK) 2, CDK4, and CDK6 (all made in mouse; Cell Signaling Technology, Inc.); retinoblastoma (Rb; made in mouse; BD Pharmingen); pRb-Ser<sup>780</sup>, p27/Kip1, Akt, and pAkt-Ser<sup>473</sup> (all made in rabbit; Cell Signaling); IRS-1 (Upstate); pIRS-1-Ser<sup>636/639</sup> (Cell Signaling); and pAkt-Ser<sup>473</sup> (mouse monoclonal; Calbiochem). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were used (Cell Signaling). Each experiment was performed in duplicates.

**Coimmunoprecipitation.** AtT-20 cells were treated with octreotide, rapamycin, and their combination for 5 min and collected in ice-cold lysis buffer [150 mmol/L NaCl, 50 mmol/L HEPES (pH 7.4), 1 mmol/L sodium orthovanadate, 2 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1% NP40]. Protein (600  $\mu\text{g}$ ) was immunoprecipitated with an antibody against IRS-1 (Upstate) or with a control rabbit IgG. Protein A-Sepharose (Amersham Pharmacia Biotech) was used as previously described (15). The immunoprecipitates were extensively washed, and protein bound to Sepharose was eluted and separated by 10% SDS-PAGE. Western blot was performed using the HRP-conjugated anti-phosphotyrosine 4G10 (Upstate), anti-phosphoserine (Zymed), or anti-IRS-1 antibody (Upstate). Coimmunoprecipitation was performed in two independent experiments and repeated using protein G-Sepharose.

**Transfection and reporter assays.** The SHP-1/C453S dominant-negative mutant (SHP-1dn; ref. 23) and the constitutively active myristoylated Akt (Myr-Akt; ref. 24) were used. Cell transfection was performed with SuperFect (Qiagen GmbH). Cells ( $3 \times 10^5$ ) were transfected for 3 h with 1  $\mu\text{g}$  of SHP-1dn plasmid, left in cell growth medium overnight, and treated with octreotide alone or in combination with rapamycin. Lysates of cells treated for 3 h were analyzed for pAkt-Ser<sup>473</sup> and pIRS-1-Ser<sup>636/639</sup> by Western blot. Each transfection experiment was performed in duplicates. To confirm the SHP-1dn and Myr-Akt incorporation, Western blot was performed for hemagglutinin (Acris).

The E2F-Luc construct (Mercury pathway profiling system, Clontech Laboratories, Inc.) has the E2F-responsive element upstream to the TATA box of the herpes simplex virus thymidine kinase promoter and the reporter gene luciferase. Cells transfected with E2F-Luc were treated with octreotide, rapamycin, and their combination for 6 h, and luciferase activity was measured by a Berthold luminometer. Transfection efficiency was determined using the Rous sarcoma virus- $\beta$ -gal construct as described previously (19). Data are expressed as the ratio of E2F relative luciferase activity (RLA) to  $\beta$ -gal activity. Each experiment was performed in triplicates.

**RNA interference.** Double-stranded small interfering RNA (siRNA) against mouse p27/Kip1 (*Cdkn1b*) was obtained from Santa Cruz Biotechnology. One scrambled siRNA (Scramble II; MWG Biotech) was used as a control.

AtT-20 cells were transfected with 100 nmol/L scrambled siRNA or siRNA against p27/Kip1 using SuperFect for 3 h and left in cell growth medium overnight to recover. The day after, they were split and distributed for proliferation assays and for RNA extraction. Cells for RNA extraction were harvested at the same time with the proliferation assay (i.e., 48 h after transfection with the siRNA). Each experiment was performed twice.

**Statistics.** All the [ $^3\text{H}$ ]TdR results were assessed by one-way ANOVA in combination with Scheffé's test.  $P < 0.05$  was considered as significant.

## Results

**Octreotide reverses rapamycin-induced increase in Akt phosphorylation.** To identify a cell model to test our hypothesis, the presence of the feedback of rapamycin on Akt phosphorylation was determined in two immortalized pituitary tumor cell lines: AtT-20 and GH3. Treatment with 1 nmol/L rapamycin increased pAkt-Ser<sup>473</sup> levels in AtT-20 cells (Fig. 1A), whereas it had no effect in GH3 cells, confirming our previous study (20). Based on these results, AtT-20 cells were used as experimental model of rapamycin-resistant cells. In these cells, addition of 1 nmol/L octreotide to rapamycin treatment decreased pAkt-Ser<sup>473</sup> levels. This observation shows that octreotide affects the feedback loop of rapamycin on Akt phosphorylation.

**Octreotide treatment inhibits the action of rapamycin on IRS-1.** The rapamycin-induced increase in pAkt-Ser<sup>473</sup> and subsequent resistance to its antiproliferative action were suggested to arise from the elimination of the negative feedback of the mTOR target p70 S6K on IRS-1 (Supplementary Fig. S1). In line with this hypothesis, herein, it is shown that treatment with octreotide increases the levels of IRS-1 detected with the phosphoserine antibody, which had been decreased by rapamycin (Fig. 1B). In contrast, octreotide suppressed IRS-1 levels detected with the 4G10 phosphotyrosine antibody (Fig. 1B), as was previously shown (14). Furthermore, rapamycin was found to decrease pIRS-1-Ser<sup>636/639</sup> levels and addition of octreotide eliminated this effect (Fig. 1C).

Octreotide was shown to transduce its effects on IRS-1 tyrosine phosphorylation through SHP-1. Transfection with a catalytically inactive SHP-1 abolished the mediated increase of octreotide in pIRS-1-Ser<sup>636/639</sup> levels (Fig. 2A) and decrease in pAkt-Ser<sup>473</sup> levels (Fig. 2B).

Octreotide treatment did not affect the suppressed p70 S6K of rapamycin (Supplementary Fig. S2). Rapamycin also decreased mTOR phosphorylation at the Ser<sup>2448</sup> and Ser<sup>2481</sup> sites.

These data show that octreotide increases serine-phosphorylated IRS-1 levels and decreases pAkt-Ser<sup>473</sup>, in a mechanism involving SHP-1, and suggest that it could improve the antiproliferative action of rapamycin.

**Antiproliferative action of the combined octreotide-rapamycin treatment in immortalized pituitary tumor cells.** Rapamycin treatment had a small antiproliferative effect in AtT-20 cells at 1 nmol/L concentration (25% growth suppression;  $P < 0.05$ ) and no effect at lower doses (Fig. 3A). In these cells, addition of 1 nmol/L octreotide to 1 nmol/L rapamycin led to 50% inhibition, as determined by both [ $^3\text{H}$ ]TdR (Supplementary Fig. S3A) and a nonradioactive colorimetric assay ( $P < 0.001$  versus vehicle control, and  $P = 0.003$  versus rapamycin alone; Fig. 3B). The antiproliferative effect of the combined treatment remained even at lower rapamycin concentrations ( $P < 0.01$ ). Octreotide alone had a mild antiproliferative effect (20% growth suppression;  $P < 0.01$ ).

To confirm the sensitivity of AtT-20 cells to the combined treatment, a soft agar colony formation assay was used. A 24-hour treatment with the combined octreotide-rapamycin treatment inhibited anchorage-independent colony formation in AtT-20 cells (Supplementary Fig. S3B).

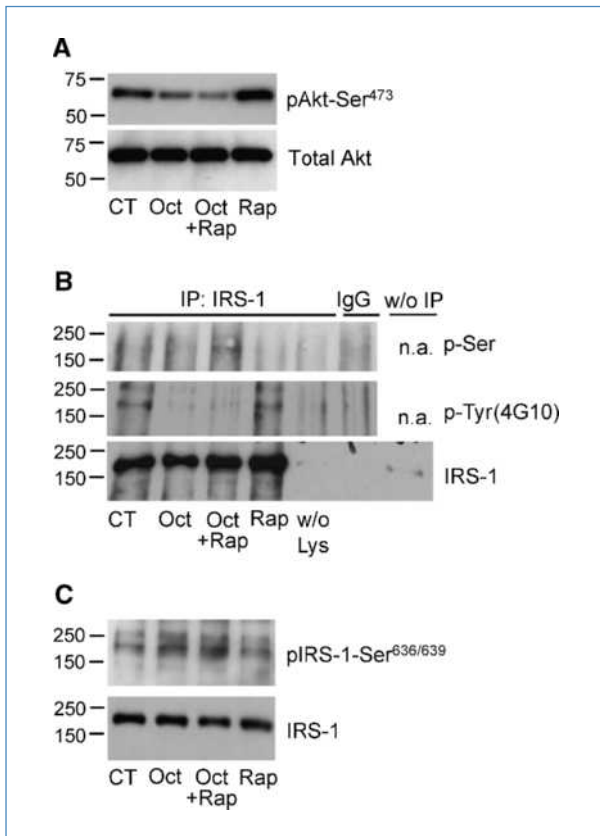
**Antiproliferative action of the combined octreotide-rapamycin treatment in human pituitary tumors in primary cell culture.** The antiproliferative efficacy of the combined treatment was tested in human NFPA in primary cell culture. In NFPA, Akt is overactivated (25) so these tumors could benefit from rapamycin treatment. Treating NFPA in primary cell culture with 1 nmol/L rapamycin decreased proliferation by <20% in 8 of 28 tumors (mean suppression of [ $^3\text{H}$ ]TdR incorporation as % of vehicle control:  $27 \pm 6$ ; Fig. 3C). Addition of 1 nmol/L octreotide further suppressed proliferation (% mean suppression:  $44 \pm 13$ ), and the suppressive effect of the combined rapamycin-octreotide treatment was significantly higher than that of rapamycin alone ( $P = 0.009$ ) in these eight tumors.

However, most of the NFPA (20 of 28) did not respond to 1 nmol/L rapamycin alone (Fig. 3C), displaying resistance to this treatment. Combined octreotide-rapamycin treatment in these tumors significantly reduced proliferation in all cases (% mean suppression:  $38 \pm 13$ ;  $P < 0.001$ ). Altogether, all 28 NFPA responded to the combined octreotide-rapamycin treatment, with [ $^3\text{H}$ ]TdR inhibition ranging from 20% to 74%.

In six tumors that provided enough cells for dose-response studies, the suppressive action of the combined octreotide-rapamycin treatment (1 nmol/L; % mean suppression:  $45 \pm 17$ ;  $P = 0.018$ ) happened after adding concentrations of rapamycin as low as 0.1 nmol/L and 0.01 to 1 nmol/L octreotide [% mean suppression:  $43 \pm 22$  ( $P = 0.037$ ) and  $42 \pm 21$  ( $P = 0.019$ ), respectively; Fig. 3D].

Single octreotide treatment did not suppress proliferation in most NFPA, similar to what has been extensively reported in previous studies. Seven of 28 NFPA responded to 1 nmol/L octreotide (% mean suppression:  $26 \pm 5$ ). Addition of 1 nmol/L rapamycin to octreotide in these seven cases had a significantly higher antiproliferative effect compared with octreotide alone (% mean suppression:  $50 \pm 18$ ;  $P = 0.007$ ; Supplementary Fig. S3C).

**Effect of combined octreotide-rapamycin treatment on cell cycle components.** Both rapamycin and octreotide are cytostatic (26–28), and the combined treatment accumulated the cells in the G<sub>0</sub>-G<sub>1</sub> phase (Supplementary Fig. S4A).



**Figure 1.** A, AtT-20 cell lysates treated with DMSO (CT), 1 nmol/L octreotide plus DMSO (Oct), 1 nmol/L rapamycin (Rap), and 1 nmol/L octreotide plus 1 nmol/L rapamycin for 3 h analyzed by Western blot using anti-Akt and anti-pAkt-Ser<sup>473</sup>. Representatives of three experiments are shown. B, AtT-20 cell lysates were immunoprecipitated with protein A-Sepharose and anti-IRS-1 or a control rabbit IgG. The immunoprecipitated fractions were analyzed by Western blot using anti-phosphoserine (p-Ser), anti-phosphotyrosine 4G10 [p-Tyr(4G10)], and anti-IRS-1. Representatives of two experiments are shown. n.a., not applicable. C, AtT-20 cell lysates treated with DMSO (CT), 1 nmol/L octreotide plus DMSO (Oct), 1 nmol/L rapamycin, and 1 nmol/L octreotide plus 1 nmol/L rapamycin for 3 h analyzed by Western blot using anti-pIRS-1-Ser<sup>636/639</sup> and anti-IRS-1. Representatives of three experiments are shown.

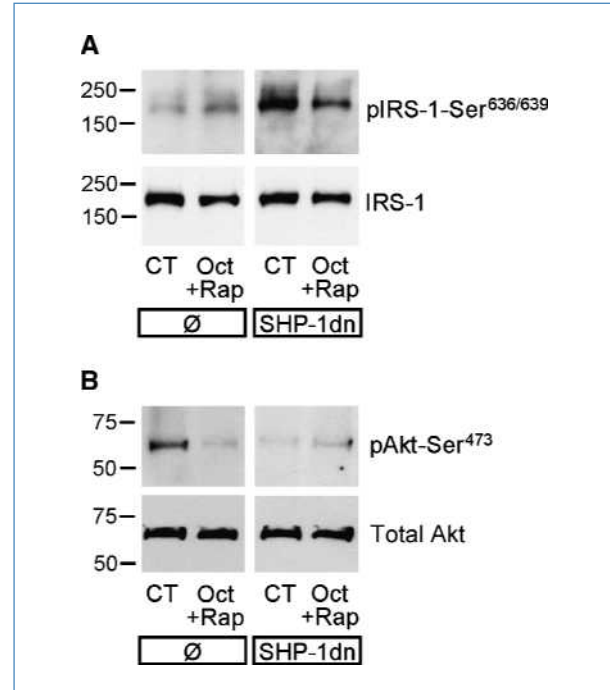
Therefore, the mechanism behind the superior antiproliferative action of their combination was sought among the proteins important for the G<sub>1</sub>-S cell cycle transition. Cell cycle progression starts with the activation of D-type cyclins and their associated kinases CDK4 and CDK6 (29). Rapamycin alone or in combination with octreotide decreased cyclin D1, cyclin D3, CDK4, and CDK6 (Fig. 4A), whereas octreotide alone had no effect and did not potentiate the effect of rapamycin, suggesting that the potent antiproliferative action of the combined treatment is not due to a more potent suppression of D-type cyclins.

The G<sub>1</sub> to S transition is primarily governed by the cyclin E/CDK2 complex (30). Single and combined rapamycin treatment decreased CDK2 protein levels (Fig. 4A). Cyclin E/CDK2 complexes act by hyperphosphorylating and inhibiting Rb. The resulting pRb dissociates from E2F transcription factors,

leading to increased E2F-driven gene transcription (31). Rapamycin decreased Rb phosphorylation levels, but addition of octreotide almost eliminated them (Fig. 4B). Furthermore, the combined octreotide-rapamycin treatment reduced E2F transcriptional activity more potently than rapamycin alone ( $P = 0.012$ ; Fig. 4C), whereas octreotide alone had a small effect, which did not reach statistical significance. E2F transcription factors drive the transcription of several genes, whose products are important for the transition to the S phase, including cyclin E. The combined octreotide-rapamycin treatment decreased cyclin E protein expression more potently than each individual treatment alone (Fig. 4D). Single octreotide treatment decreased cyclin E, similar to what was reported for the somatostatin analogue RC-160 in CHO/SSTR2 cells (23) and somatostatin in pancreatic acinar cells (32). Treatment with the protease inhibitor MG132 did not affect cyclin E suppression after single or combined octreotide and rapamycin treatment (Supplementary Fig. S4B).

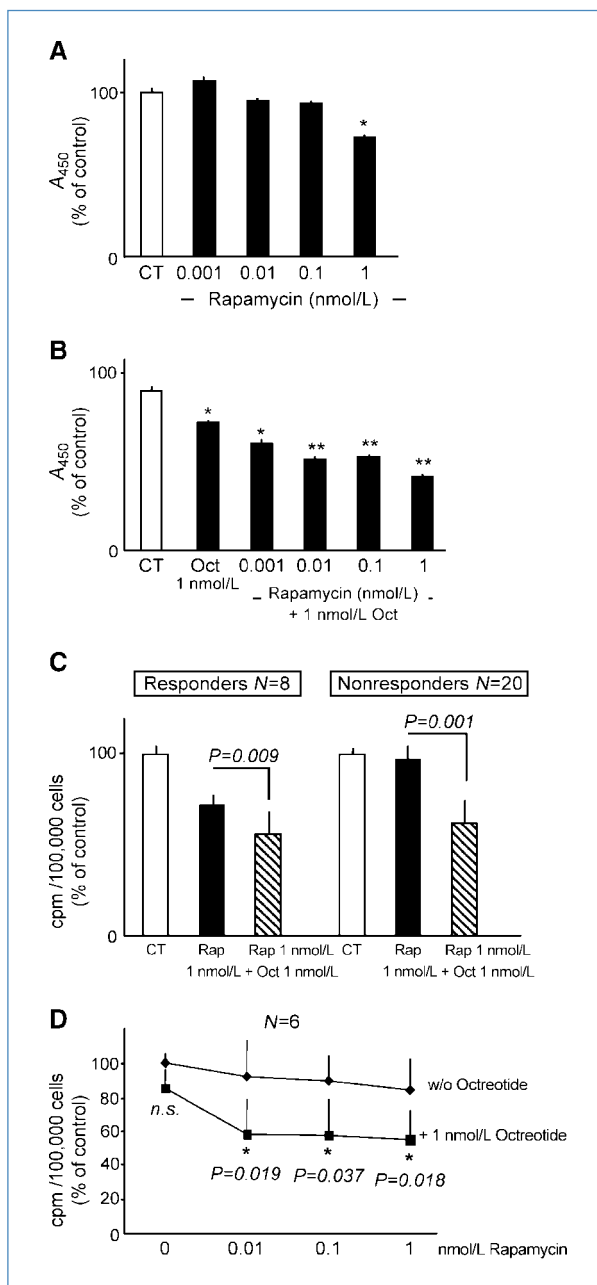
These results suggest that the combined treatment decreases E2F transcriptional activity and cyclin E transcription, prohibiting the tumor cells from entering the S phase of the cell cycle. However, the rapamycin-induced decrease in CDK2 levels alone cannot explain the better suppressive effect of the combined octreotide-rapamycin treatment on E2F transcriptional activity and cyclin E transcription.

**The combined octreotide-rapamycin treatment upregulates p27/Kip1.** The primary regulator of the cyclin E/CDK2



**Figure 2.** Control-transfected and SHP-1dn-transfected AtT-20 cell lysates treated with DMSO (CT) and 1 nmol/L octreotide plus 1 nmol/L rapamycin analyzed by Western blot using anti-pIRS-1-Ser<sup>636/639</sup> and anti-IRS-1 (A) and anti-Akt and anti-pAkt-Ser<sup>473</sup> (B). Representatives of two independent transfection experiments are shown.





**Figure 3.** Antiproliferative effect of 0.001, 0.01, 0.1, and 1 nmol/L of rapamycin alone (A) or in combination with 1 nmol/L octreotide (B) in AtT-20 cells. Treatments were performed in 10% FCS-DMEM. Cell proliferation was determined after 24-h treatment. Data are presented as percentage of each vehicle-treated control. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ . Each measurement was done in four independent wells. C, [ $^3$ H]TdR uptake inhibition in human NFPAs in primary cell culture treated with 1 nmol/L rapamycin and 1 nmol/L rapamycin plus 1 nmol/L octreotide; mean of eight NFPAs that were responsive to single rapamycin treatment and mean of 20 NFPAs nonresponsive to the antiproliferative action of rapamycin. D, dose curve study on 6 of 28 NFPAs treated with 0.01, 0.1, and 1 nmol/L of rapamycin alone or in combination with 1 nmol/L octreotide. All treatments were performed in 10% FCS-DMEM supplemented with D-valine. Data are expressed as percentage of vehicle-treated control. Points, mean; bars, SE. Each measurement was done in four independent wells.

complex is the cyclin kinase inhibitor p27/Kip1. Single octreotide and rapamycin treatments increased p27/Kip1 protein levels, similar to what was described in previous studies (23, 33), but their combination had a stronger effect on p27/Kip1 transcript (Supplementary Fig. S5A) and protein levels (Fig. 5A). These data suggest that increased p27/Kip1 transcription is an adjuvant mechanism by which the combined treatment exerts its superior antiproliferative action. Indeed, knocking down p27/Kip1 abolished the antiproliferative action of the combined octreotide-rapamycin treatment without affecting that of the single rapamycin treatment (Supplementary Fig. S5B). Furthermore, adding octreotide to rapamycin treatment in the GH3 cells, which do not express p27/Kip1 (34), did not enhance the antiproliferative effect of rapamycin (Supplementary Fig. S5C).

The stronger effect of the combined treatment on p27/Kip1 indicates that the two drugs may induce p27/Kip1 through different pathways. The stimulatory effect of octreotide on basal and rapamycin-induced p27/Kip1 was not observed in AtT-20 cells transfected with SHP-1dn (Fig. 5B) or with a constitutively active Myr-Akt (Fig. 5C), whereas neither intervention affected rapamycin. Instead, the stimulatory action of rapamycin on p27/Kip1 was blocked after inhibiting glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) with 20 mmol/L lithium chloride (Supplementary Fig. S5D) or 14  $\mu$ mol/L of the selective inhibitor SB415286 (Fig. 5D).

## Discussion

Resistance to cytotoxic and cytostatic treatments is a common limitation in cancer treatment. Rapamycin resistance was in part attributed to the rapamycin-induced increase in pAkt-Ser<sup>473</sup> levels, which was documented *in vitro* and *in vivo* on biopsies of patients treated with the rapalog RAD001 (7) and was found to derive from the elimination of the negative feedback loop of the mTOR target p70 S6K on the PI3K pathway. In the present study, we sought to suppress the PI3K pathway upstreams with a somatostatin analogue that was previously shown to target this pathway (14, 15) to restore the control of Akt and therefore induce sensitivity to rapamycin treatment.

The serine/threonine kinase p70 S6K phosphorylates IRS-1 at serine residues, causing its inactivation. Rapamycin, by inhibiting mTOR, decreases p70 S6K activity and reduces serine IRS-1 phosphorylation, leading to its activation. Octreotide was previously reported to inhibit IRS-1 by decreasing its tyrosine phosphorylation levels through the phosphotyrosine phosphatase SHP-1 (14). Herein, we show that it also increases IRS-1 serine phosphorylation levels and, in particular, pIRS-1-Ser<sup>636/639</sup>, which is the site primarily phosphorylated by p70 S6K. Interestingly, this effect was also SHP-1 dependent. Tyrosine-phosphorylated IRS-1 was previously reported to bind to another phosphotyrosine phosphatase (PTP2C) and to get dephosphorylated and inhibited through this association (35). Whether SHP-1 plays a similar role in regulating IRS-1 remains to be determined. Nevertheless, it is noteworthy that the basal levels of pIRS-Ser<sup>636/639</sup> in the cells transfected with the catalytically inactive SHP-1 were higher

than those of the control cells, suggesting that changes in tyrosine phosphorylation undermine the IRS-1 serine phosphorylation status.

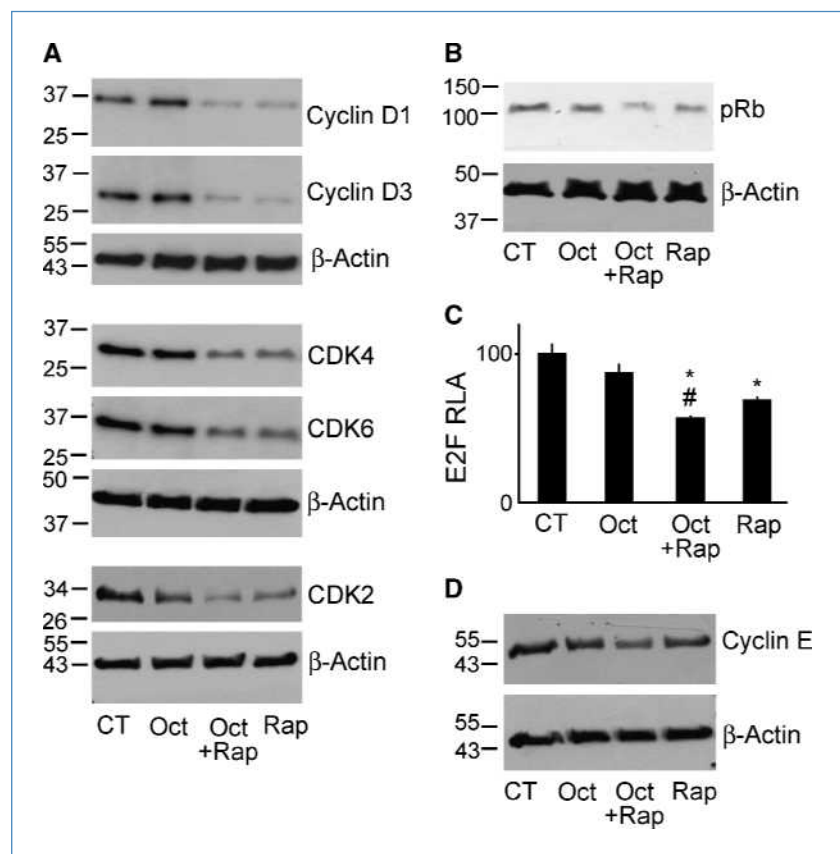
Single and combined rapamycin treatment decreased mTOR phosphorylation at both Ser<sup>2448</sup> and Ser<sup>2481</sup>, similar to previous studies (36). However, the rapamycin-suppressed p70 S6K phosphorylation was not altered by cotreatment with octreotide, indicating that the effect of octreotide on rapamycin-induced IRS-1 is not due to an action of the drug on p70 S6K.

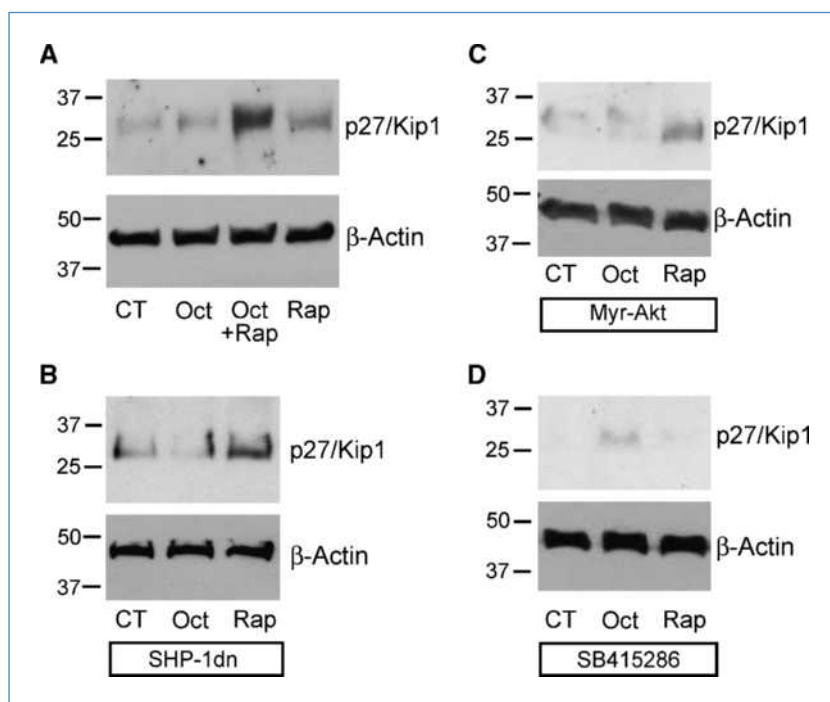
Octreotide enhanced the antiproliferative action of rapamycin in immortalized mouse pituitary tumor cells that responded poorly to the antiproliferative action of rapamycin and in human pituitary tumors in primary cell culture. The present study focused on NFPA, which were shown to display high levels of activated Akt compared with normal pituitary or with other pituitary tumor types and could therefore be a good candidate for treatment with rapamycin (25). In our study, more than half of the cases could not respond to rapamycin, suggesting resistance to this treatment. It is noteworthy that addition of octreotide improved the antiproliferative action of rapamycin not only in rapamycin-resistant tumors but also in rapamycin responders. The improvement of the antiproliferative action of rapamycin by octreotide was not due to its own growth-inhibiting effect in NFPA because only few cases responded to single octreotide treatment, reflecting *in vitro* (37) and *in vivo* (38–40) observations.

Interestingly, the superior effect of the combined treatment was present even at low rapamycin concentrations. These findings suggest that the same antiproliferative effect can be achieved at lower pharmacologic doses of the rapamycin analogs, which is of clinical importance considering their relative toxicity at high doses (41).

Searching for mechanisms mediating the superior antiproliferative effect of the combined treatment downstream to IRS-1/Akt suppression, we focused on the G<sub>1</sub>-S cell cycle transition because the combined treatment arrested the cells in the G<sub>0</sub>-G<sub>1</sub> phase. Rapamycin decreased cyclin D1, cyclin D3, CDK4, CDK6, and CDK2 similar to what was described in previous studies (42–44). Addition of octreotide did not potentiate this action, but it nevertheless suppressed Rb phosphorylation, E2F transcriptional activity, and the E2F transcriptional target cyclin E more potently than rapamycin treatment alone. Both octreotide and rapamycin were previously shown to suppress cyclin E (20, 32, 45, 46), so this finding is not surprising. However, these data collectively indicate a strong inhibitory action on CDK2 activity, upstream to cyclin E, which cannot be explained only by the rapamycin-induced decrease in CDK2. Cyclin E/CDK2 activity is negatively regulated primarily by the cell-dependent kinase inhibitor p27/Kip1, which was previously reported to be upregulated by octreotide and rapamycin (23, 33). Nevertheless, the combination of the two drugs strongly

**Figure 4.** AtT-20 cell lysates treated with DMSO (CT), 1 nmol/L octreotide plus DMSO (Oct), 1 nmol/L rapamycin, and 1 nmol/L octreotide plus 1 nmol/L rapamycin for 24 h analyzed by Western blot using anti-cyclin D1, anti-cyclin D3, anti-CDK4, anti-CDK6, and anti-CDK2 (A) and anti-pRb-Ser<sup>780</sup> (B). Representative  $\beta$ -actin is shown. Cells were serum deprived for 24 h before treatment. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown. C, effect of 6-h treatment with 1 nmol/L octreotide, 1 nmol/L rapamycin, and their combination on E2F-mediated transcription in AtT-20 cells transfected with E2F-Luc. Results are shown as E2F RLA to  $\beta$ -gal ratio. Each experiment was repeated twice. \*,  $P < 0.05$ ; #,  $P = 0.012$ . D, Western blot for cyclin E. The membrane blotted for CDK2 (shown in A) was used after stripping with 100 mmol/L Tris-HCl (pH 2.0).





**Figure 5.** Western blot for p27/Kip1 on AtT-20 cell lysates treated with DMSO (CT), 1 nmol/L octreotide plus DMSO (Oct), 1 nmol/L rapamycin, and 1 nmol/L octreotide plus 1 nmol/L rapamycin for 24 h (A); SHP-1dn-transfected (B) and Myr-Akt-transfected (C) AtT-20 cell lysates treated with DMSO (CT), 1 nmol/L octreotide plus DMSO (Oct), and 1 nmol/L rapamycin; and AtT-20 cell lysates treated as in B and C in the presence of the GSK3 $\beta$  inhibitor SB415286 (14  $\mu$ mol/L; D).

enhanced p27/Kip1 expression, providing with an additional mechanism for its superior antiproliferative action downstreams at cell cycle level. Indeed, cells in which p27/Kip1 was knocked down or do not express p27, such as the GH3 cells, failed to better respond to the combined octreotide-rapamycin treatment compared with the individual treatments.

The extent of the p27/Kip1 upregulation after the combined octreotide-rapamycin treatment suggests that the two drugs may use different pathways to induce this cell cycle inhibitor. The action of octreotide on p27/Kip1 was SHP-1 dependent, reflecting previous studies in pituitary tumor cells (15). Interestingly, Akt overexpression also abolished the stimulatory effect of octreotide, indicating that Akt overactivation may block the cytostatic action of octreotide in pituitary tumor cells. Indeed, Akt overexpression eliminated the antiproliferative action of octreotide in the AtT-20 pituitary tumor cells (Supplementary Fig. S6). These data raise the interesting possibility that Sst2-expressing tumors with overactivated Akt may be more resistant to the antiproliferative action of somatostatin analogues that target mainly this receptor, such as octreotide and lanreotide. In contrast, the effect of rapamycin did not depend on SHP-1 or Akt but on GSK3 $\beta$ , which, as a downstream target of p70 S6K (47), is activated by rapamycin (48).

Inhibition of the PI3K pathway upstreams could facilitate tumor cells to respond to rapamycin by inhibiting mitogenic and survival signaling and cell cycle progression. In previous studies, addition of small-molecule inhibitors against PI3K (12) or insulin-like growth factor-I receptor (7) abolished the rapalog-induced Akt phosphorylation and improved its efficacy. In the present study, we chose to target the PI3K

pathway with a biological acting on a G protein-coupled receptor. Sst2 is present on neuroendocrine tumors, and targeting this receptor with somatostatin analogs, such as octreotide, constitutes a first-line pharmacologic treatment for these tumors (16). Using a Sst2 agonist to target the PI3K pathway and improve the antitumor action of rapamycin could ensure a tumor-specific action. While the present study was ongoing, the combination of octreotide long-acting repeatable and the rapalog RAD001 (everolimus) went in phase II clinical trials for the treatment of advanced neuroendocrine tumors and was shown to be well tolerated with promising antitumor activity (49). Our observation that octreotide can sensitize tumor cells to lower concentrations of rapamycin indicates that a beneficial effect can be expected after treatment with lower doses of the drug, which is important in case of adverse side effects. Clinical trials are needed to address the efficacy of the combined treatment in patients with NFPA and other neuroendocrine tumors. Interestingly, two recent studies in gastroenteropancreatic neuroendocrine tumor cell lines failed to show improvement of the antiproliferative action of rapamycin after addition of octreotide (50, 51). In our study, the same outcome was observed in the GH3 pituitary tumor cells and was attributed to the lack of p27/Kip1 in these cells (34). Intrinsic differences in each individual tumor are suspected to be responsible for these discrepancies, and future studies are essential to identify factors that may contribute to resistance to the beneficial effects of the combined treatment.

Summarizing, in the present study, we showed that octreotide blocks one of the mechanisms that are suspected to be responsible for rapamycin resistance in a mechanism involving SHP-1, decreased IRS-1 tyrosine, and increased IRS-1

serine phosphorylation. These events lead to suppressed pAkt-Ser<sup>473</sup> levels and increased p27/Kip1, which blocks CDK2 activity and eventually cell cycle progression. The herein described mechanistic background indicates that adding somatostatin analogues to rapamycin analogue treatment could efficiently limit growth not only in NFPA but also in other somatostatin receptor-expressing tumor types for which treatment with mTOR inhibitors is indicated.

## Disclosure of Potential Conflicts of Interest

G.K. Stalla: commercial research grant, Novartis Pharma GmbH. M. Theodoropoulou: independent consultant, Novartis Pharmaceuticals Corp. The other authors disclosed no potential conflicts of interest.

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## Acknowledgments

We thank Dr. Klaus Molle (Biozentrum, Basel, Switzerland) for providing the Myr-Akt plasmid.

## Grant Support

Deutsche Forschungsgemeinschaft grant TH 901/1-3 (M. Theodoropoulou) and Novartis Pharma GmbH (Nürnberg, Germany) research grant (G.K. Stalla).

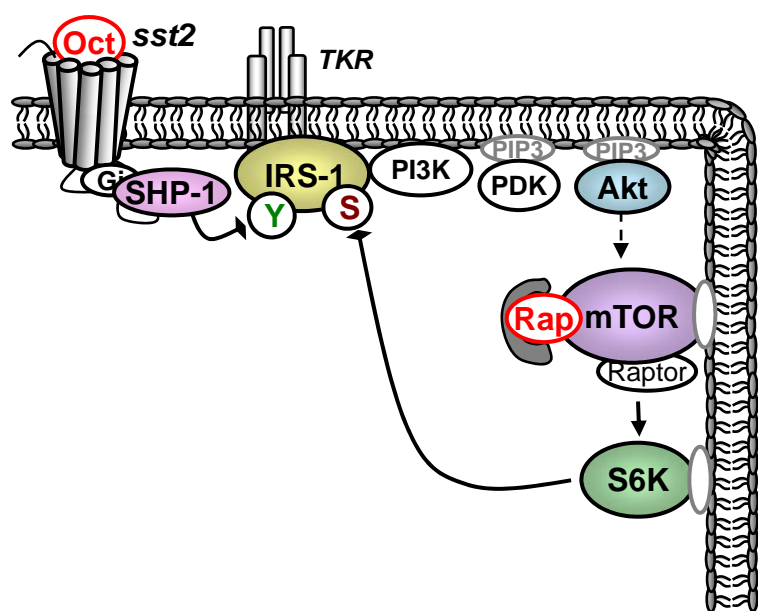
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Received 8/8/09; revised 10/23/09; accepted 11/5/09; published OnlineFirst 1/12/10.

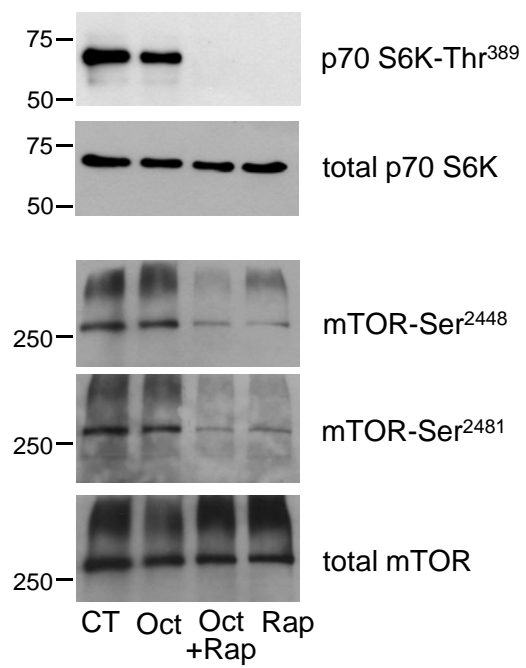


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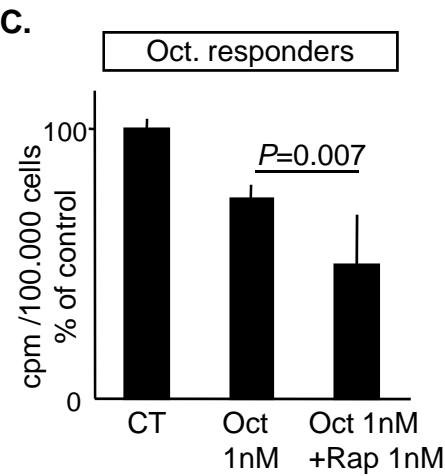
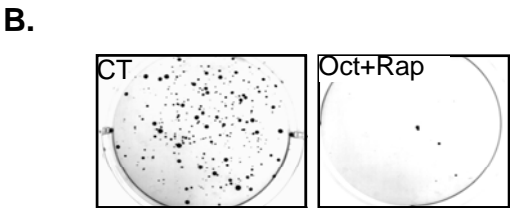
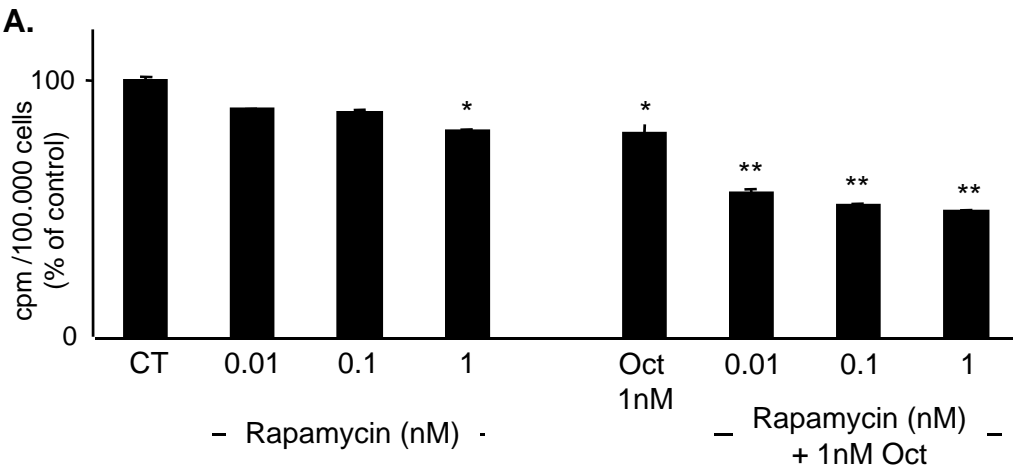
Supplementary Figure 1



## Supplementary Figure 2

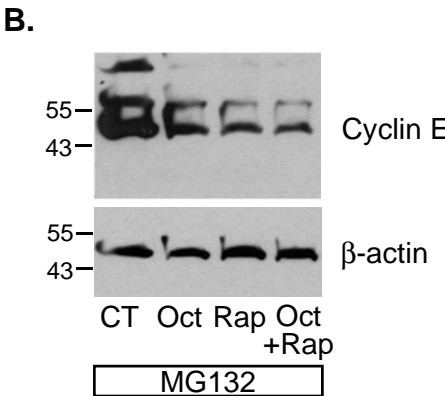
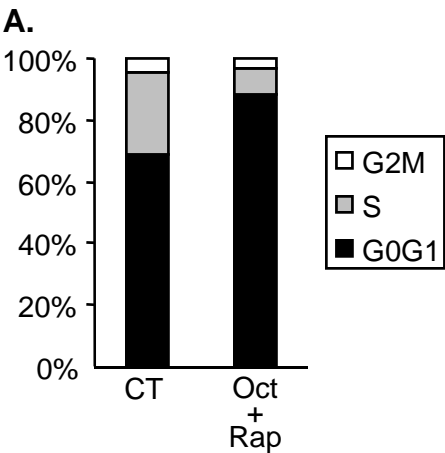


Supplementary Figure 3



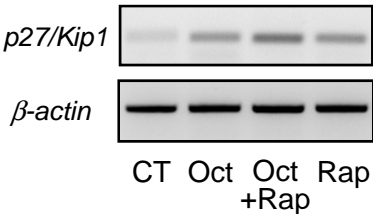


Supplementary Figure 4

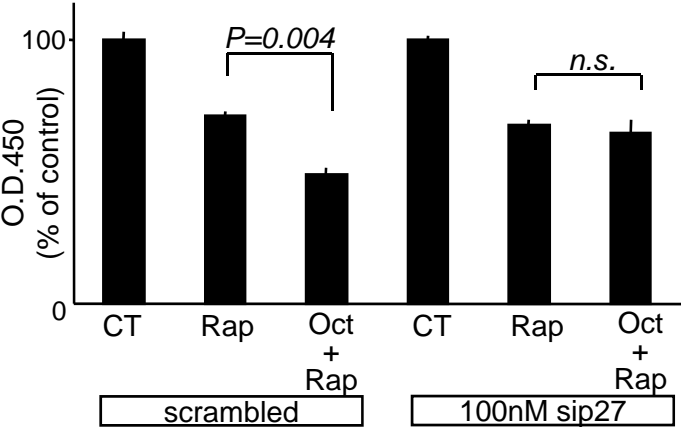


Supplementary Figure 5

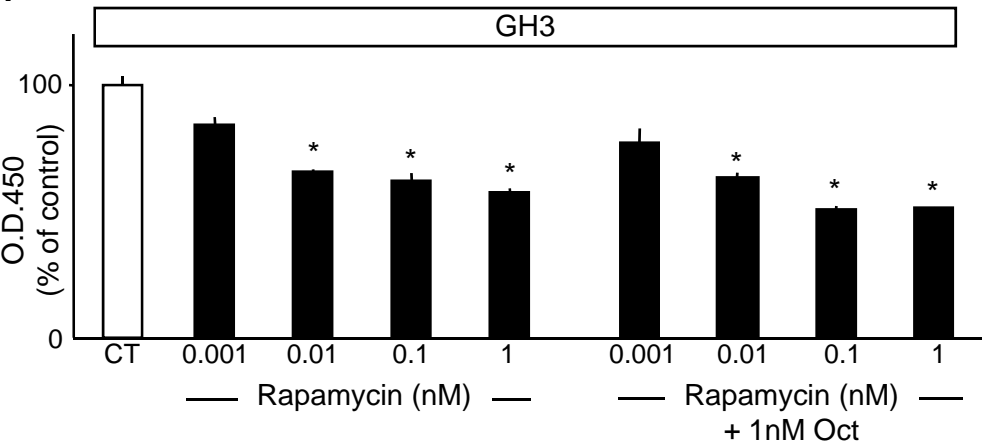
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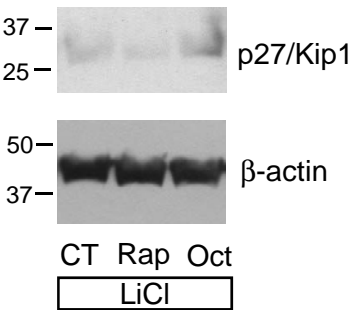
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Supplementary Figure 6

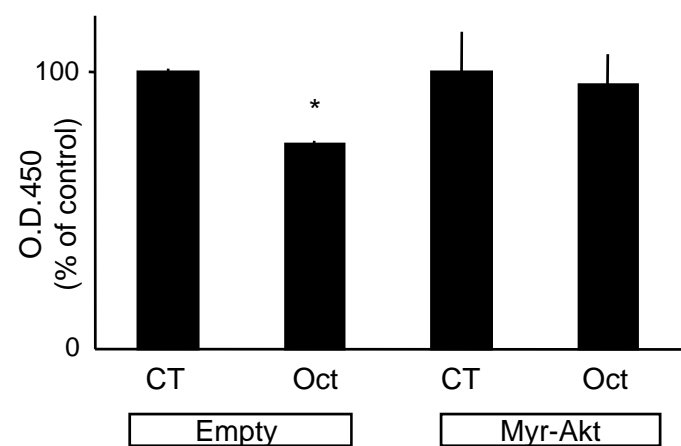


Fig. S1. Scheme showing how octreotide and rapamycin affect the PI3K pathway upstreams at IRS-1 level. In brief, ligand bound, activated tyrosine kinase receptors (TKR), such as, IGF-I and insulin receptors, tyrosine (Y) phosphorylate and activate IRS-1. Activated IRS-1 acts as an adaptor recruiting class I PI3K, which produces phosphatidylinositol-3,4,5-triphosphates (PIP3) and subsequently bring PDK1 and Akt on the membrane, where they can be activated. This pathway switches off by a negative feedback originated downstreams to Akt-activated mTOR. The serine/threonine kinase p70 S6K phosphorylates IRS-1 at serine residues (S), which act in inhibitory fashion. Rapamycin (Rap) by inhibiting mTOR abolishes this negative feedback loop leading to overactivation of the PI3K pathway, determined by increased phosphorylated Akt levels. Addition of the sst2 ligand octreotide (Oct), which activates the phosphotyrosine phosphatase SHP-1, results in dephosphorylation of IRS-1 at the tyrosine residues and its subsequent inhibition. Diamond-ended arrows: inhibition; solid arrows: direct action; dashed arrows: indirect action.

Fig. S2. AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin analyzed by Western blot using anti-p70 S6K-Thr389, -total p70 S6K, -mTOR-Ser2448, -mTOR-Ser2481, and -total mTOR (all made in rabbit; Cell Signaling).

Fig. S3. (A) 3H-TdR uptake inhibition in AtT-20 cells treated with 0.01, 0.1 and 1nM rapamycin alone or in combination with 1nM octreotide. Treatments were performed in 10% FCS-DMEM. Cell proliferation was determined after 24 hours treatment. Data are presented as percentage of each vehicle-treated control. \*:  $P < 0.05$ , \*\*:  $P < 0.001$ . (B) AtT-20 cells treated for 24 hours with vehicle (CT) or 1nM octreotide plus 1nM rapamycin and seeded in soft agar. After 20 days colonies were stained with MTT and photographed. (C) 3H-TdR uptake inhibition in human NFPA in primary cell



culture treated with 1nM octreotide and 1nM rapamycin plus 1nM octreotide; mean of seven NFPA that were responsive to single octreotide treatment.

Fig. S4. (A) AtT-20 cells were incubated with vehicle or 1nM rapamycin plus 1nM octreotide for 24 hours and were analyzed by flow cytometry. (B) AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin in the presence of the protease inhibitor MG132 (10 $\mu$ M) for 24 hours analyzed by Western blot using anti-cyclin E.

Fig. S5. (A) p27/Kip1 expression in AtT-20 cells after 24 hour treatment with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin as determined by RT-PCR. (B) Effect of p27/Kip1 RNA interference on the antiproliferative action of the combined rapamycin-octreotide treatment; cell proliferation was determined in cells transfected with 100nM scrambled or p27/Kip1 siRNA, treated with 1nM rapamycin alone or in combination with 1nM octreotide for 24 hours. \*:  $P < 0.05$ . (C) Antiproliferative effect of 0.001, 0.01, 0.1 and 1nM rapamycin alone or in combination with 1nM octreotide in GH3 cells. Treatments were performed in 10% FCS-DMEM. Cell proliferation was determined after 24 hours treatment. Data are presented as percentage of each vehicle-treated control. \*:  $P < 0.05$ . (D) Western blot for p27/Kip1 on AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), and 1nM rapamycin for 24 hours in the presence of the GSK3 $\beta$  inhibitor lithium chloride (LiCl; 20mM).

Fig. S6. Effect of constitutive active Akt on octreotide's antiproliferative action; cell proliferation was determined in cells transfected with 0.5ng empty vector or myr-Akt, treated with 1nM octreotide for 24 hours. \*:  $P < 0.05$ .